# Simultaneous Quantification of *Aspergillus flavus*/*A. parasiticus* and Aflatoxins in Peanuts

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**Abstract**

A method was developed for simultaneous quantification of *Aspergillus flavus/A. parasiticus* and aflatoxins in peanuts. Peanut samples were ground with an equal weight of water in a vertical cutter mixer to produce a slurry. Separate subsamples were taken for dilution plating to determine total colony forming units (CFU)/g of *A. flavus/A. parasiticus* and for LC analysis to determine aflatoxin concentrations. Dry-grinding peanuts for homogenization of aflatoxins produced high temperatures that killed most of the *A. flavus/A. parasiticus* propagules. Addition of water to produce a slurry kept the temperature from rising above levels which killed the fungi. A 7-min grind time provided optimal homogenization for both the fungi and aflatoxins, so long as the temperature of the slurry did not exceed 45° C. In the analysis of 60 shelled peanut samples, total aflatoxin concentrations ranged from 0 to 10,000 ng/g and total *A. flavus/A. parasiticus* ranged from $1.4 \times 10^3$ to $3.2 \times 10^6$ CFU/g. Regression analysis showed a significant positive correlation (P < 0.0001) between the quantities of *A. flavus/A. parasiticus* and aflatoxin ($R^2 = 0.82$).

**Introduction**

Aflatoxins are potent hepatotoxic, carcinogenic metabolites produced by *Aspergillus flavus* Link, *A. parasiticus* Speare, and *A. nomius* Kurtzman et al. (1-3). Aflatoxin contamination of peanuts and other commodities results primarily from invasion and proliferation by *A. flavus*, although *A. parasiticus* can be an important contributor to overall aflatoxin contamination, particularly in peanuts (4). *A. nomius* is relatively rare and is not considered an important species in the contamination of commodities with aflatoxins (5). Because of the toxicity and carcinogenicity of aflatoxins, contaminated commodities destined for human or animal consumption pose a serious health hazard and are, therefore, closely monitored and regulated (6, 7). Apart from its effect on health, aflatoxins also impact the agricultural economy through the loss of produce and the time and costs involved in monitoring and decontamination efforts (8).

Infection of peanuts by *A. flavus* and *A. parasiticus* can occur in the field as peanuts are forming and maturing, but infection does not necessarily result in aflatoxin contamination because growth of the fungi must also occur. When peanut plants receive adequate water, either through rainfall or supplemental irrigation, and are essentially healthy, peanuts produce phytoalexins in response to fungal infection, which inhibit proliferation by the invading fungi (9, 10) . However, when plants are exposed to severe stress as a result of extended drought, peanuts dehydrate in the soil and lose the ability to produce phytoalexins (11). Under these conditions, invading fungi such as *A. flavus* have the opportunity to grow and produce aflatoxins (11).

The relationship between *A. flavus/A. parasiticus* infection and aflatoxin contamination of peanuts is not well

understood because in some studies, high infection percentages were associated with no aflatoxin contamination, and conversely, relatively low infection percentages were associated with high levels of aflatoxin (4, 12-14). In those studies, infection percentages were usually determined by surface disinfesting a quantity of individual seeds, plating them on an agar medium, and counting the seeds from which *A. flavus* and *A. parasiticus* colonies emerged. By necessity, aflatoxin analyses had to be carried out on separate samples of peanuts. Results of such studies often have been confusing, particularly when a high percentage of seeds is infected but no aflatoxin is found. Therefore, it has been impossible to consistently correlate *A. flavus/A. parasiticus* infection with aflatoxin contamination.

In recent years a technique has been developed for biological control of aflatoxin contamination of peanuts (15, 16). Nontoxigenic strains of *A. flavus* and *A. parasiticus* are applied to soil around developing peanut plants, and through competitive exclusion, the applied strains preferentially invade peanuts exposed to late-season drought stress resulting in reductions in aflatoxin contamination ranging from 70-99%. However, in many of these studies, the correlation between the type of *A. flavus/A. parasiticus* (toxigenic vs. nontoxigenic) infecting peanuts and the amount of aflatoxin produced has not always been clear (unpublished data). These studies also used plating of surface-disinfested peanuts, which gave no indication of the nature of growth by specific strains.

In order to better understand the relationship between *A. flavus* colonization of peanuts and aflatoxin contamination, a method was needed that would allow for quantification of the amount and type of *A. flavus/A. parasiticus* present as well as the amount of aflatoxin in the same samples of peanuts. The purpose of this paper is to report:

(1) a method for simultaneous quantification of *A. flavus/A. parasiticus* and aflatoxin in peanuts, and (2) the correlation between the quantities of *A. flavus/A. parasiticus* and aflatoxin in 60 peanut samples.

**Experimental**

*Apparatus*

**(a)** *Vertical cutter mixer (VCM).* - Model RSI6Y-1 (Robot Coupe USA, Jackson, MS); model UM-12 (Stephan Machinery Corp., Columbus, OH).

**(b)** *Digital thermometer.* - Electro-therm model TM99A (Cooper Instrument Corp, Middlefield, CT).

**(c)** *Blender for aflatoxin extraction.* - Commercial model 31BL41 (Waring) operated at high speed (22,000 rpm) with 1 qt. glass jar.

**(d)** *Blender for fungal dispersal and dilution.* - Same as **(b)**, but operated at low speed with 1 L stainless steel jars, autoclaved between samples.

**(e)** *Variable autotransformer.* - 10 A, 1.4 KVA (Staco Energy Products, Dayton, OH).

**(f)** *Liquid chromatograph.* - Pump, Model 515 LC (Waters); autosampler, Model 717 plus (Waters); fluorescence detector, Model RF-551 (Shimadzu); class VP Chromatography Data System, version 4.2 (Shimadzu); photochemical reactor, "PHRED" (Aura Industries, New York, NY); column, Nova-PAK $C_{18}$ (150 × 3.9 mm; 4 μ; Waters); mobile phase: water-methanol-1-butanol (1400 + 720 + 25, v/v/v); operating conditions: column temperature 38° C; flow rate 0.8 mL/min; injection volume 20 μL; detector wavelength, 365 nm (excitation) and 440 nm (emission).

*Reagents and materials*

**(a)** *Extraction solvent.* - ACS-grade methanol (Fisher).

**(b)** *Solvents for LC.* - LC-grade methanol, 1-butanol (Fisher). LC-grade water was prepared with 4-bowl Milli-Q Water

System, Model ZD20 (Millipore).

**(c)** *Aflatoxin standard for LC.* - Prepared from crystals of $B_1$, $B_2$, $G_1$, and $G_2$ (Sigma) according to AOAC Official Method, 970.44 (17). After spectrophotometric determination of individual aflatoxin concentrations, appropriate amounts of individual standards were combined to produce an LC standard containing 5 ng of $B_1$ and $G_1$ and 1.5 ng of $B_2$ and $G_2$ per mL of injection solvent.

**(d)** *LC injection solvent.* - LC grade methanol-water (62 + 38, v/v) plus 0.1% acetic acid.

### Procedure

Prepare a peanut-water slurry by grinding shelled peanuts with an equal weight of tap water in a VCM of appropriate size for 7 min. To obtain better homogenization, the size of the VCM bowl should be such that it is ≥ half full with the peanut-water mixture. For quantification of *A. flavus/A. parasiticus*, transfer a 200-g subsample of the slurry to an autoclaved, stainless steel blender jar and add 200 mL of water. Blend the diluted slurry for 1 min at low speed (autotransformer operated at 30% of maximum output). Plate serial dilutions on modified dichloran-rose bengal medium (5, 18) and incubate for 2-3 d at 37° C. For quantification of aflatoxins, transfer a separate 200-g subsample of the original slurry to a 1 qt. glass blender jar, add 400 mL of methanol, and blend at high speed for 1 min. Carry out sample cleanup and LC analysis as described by Dorner and Cole (19), or use other suitable aflatoxin quantitative method. A schematic of the procedure is presented in Figure 1.

### Effect of dry grinding peanuts on quantity and variability of *A. flavus/A. parasiticus and aflatoxin*

A study of the variability of aflatoxin in peanut subsamples produced with different mills (20) showed that a grind time of about 6-7 min was necessary to produce acceptably
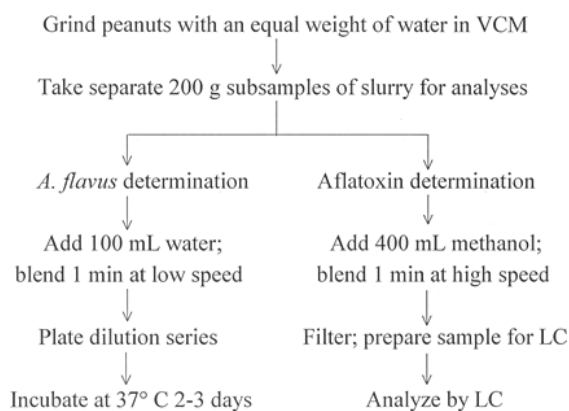


**Figure 1**. Schematic of the peanut-water slurry procedure used to quantify both *A. flavus/A. parasiticus* and aflatoxins in peanuts.

low variability among subsamples taken from a single ground sample. It is possible to dilution plate subsamples of peanuts ground in a VCM to determine the quantity of *A. flavus/A. parasiticus*, but grinding peanuts in a VCM for such a length of time produces relatively high temperatures that could kill fungi present. Therefore, an experiment was conducted to determine the effect of grind time on the quantity of *A. flavus/A. parasiticus* and aflatoxin found in subsamples. A 1.5 kg sample of shelled peanuts was ground in the RSI6Y-1 VCM for increasing 1 min intervals between 1 and 7 min. The temperature was measured with the digital thermometer and five 50-g subsamples were taken for analysis at each time interval. For fungal counts, a water slurry was prepared by homogenizing each 50-g subsample in 150 mL of water at low speed and dilution plating 1 mL of slurry. To quantify aflatoxins, 225 mL of methanol was added to the peanut-water slurry and extracted at high speed followed by LC analysis.

### Effect of grinding peanuts with water on temperature of ground samples

To determine the effect of adding water prior to grinding on the temperature of the resulting slurry in comparison with dry grinding, three 1.5 kg samples of peanuts

were ground alone and with 1.5 kg of water in the RSI6Y-1 VCM for 7 min with temperature being measured every 1 min.

*Effect of slurry temperature on survivability of* A. flavus/A. parasiticus

The temperature of the peanut-water slurry at different grind times is somewhat dependent on the total mass of the slurry and the particular VCM being used. For example, smaller samples ground in the Robot Coupe VCM have a higher temperature for a given grind time than do larger samples ground in the Stephan VCM. However, fungal survivability during grinding is more dependent on temperature than on grind time. Therefore, the effect of slurry temperature on survivability of *A. flavus/A. parasiticus* was determined by grinding 1.5 kg of peanuts with 1.5 kg of water in the RSI6Y-1 VCM and taking subsamples for dilution plating at 5° C increments beginning when the slurry temperature reached 35° C and ending at 60° C. Three subsamples were taken at each temperature and the experiment was conducted with two separate samples of peanuts from the same lot.

*Effect of slurry grind time on* A. flavus/A. parasiticus *and aflatoxin subsample variability*

In order to determine the effect of slurry grind time on *A. flavus/A. parasiticus* and aflatoxin subsampling variability, a single large sample (3 kg peanuts + 3 kg water) was ground in the larger capacity UM-12 VCM. Five separate 100-g subsamples were collected for *A. flavus/A. parasiticus* and aflatoxin quantitation at 1, 3, 5, and 7 min. Subsample size was reduced in this experiment so that an adequate amount of material remained in the VCM after each time interval for further grinding and subsampling.

*Correlation between* A. flavus/A. parasiticus *and aflatoxin*

The previously described procedure was used in the analysis of 60 samples of peanuts produced during crop year 1999 in southwestern Georgia. Samples were obtained from a variety of farmers' stock and shelled stock lots to represent a wide variety of production and storage conditions. Crop year 1999 was characterized by widespread late-season drought; therefore, many lots were suspected to contain high concentrations of aflatoxin. To reduce sampling error as much as practical, large samples averaging 3.6 kg of shelled peanuts were processed with an equal weight of water in the UM-12 VCM. Samples were ground for 7 min, and subsamples were analyzed for *A. flavus/A. parasiticus* and aflatoxin as depicted in Figure 1.

*Statistics*

Aflatoxin concentrations were log transformed prior to statistical analysis when necessary to normalize distributions. Data were subjected to analysis of variance and regression analysis using SigmaStat for Windows Version 1.0 (Jandel Corporation, San Rafael, CA). Multiple comparisons were made with the Student-Newman-Keuls method.

## Results

*Effect of dry grinding peanuts on quantity and variability of* A. flavus/A. parasiticus *and aflatoxin*

Results of the experiment showed that significantly less *A. flavus/A. parasiticus* was recovered beginning with the 3 min grind time, when the temperature rose from 42.0 to 55.1° C (Table 1). The density of *A. flavus/A. parasiticus* continued to decrease as the grind time and temperature increased until after 5 min, when the density did not change significantly. Approximately 99% of the viable *A. flavus/A. parasiticus* present after 1 min was apparently killed by the high temperature at 5 min. On the other hand, aflatoxin concentrations showed no clear pattern as a direct result of time and increasing temperature. The second highest aflatoxin concentrations was found after grinding for 6 min when the temperature had

**Table 1. Quantity and variability of *A. flavus/A. parasiticus* and aflatoxin associated with subsamples taken from VCM-ground peanuts (no water) at different time intervals**

| Grind Time (min) | Temp (° C) | A. flavus / A. parasiticus CFU/g | CV (%) | Aflatoxin [a] ng/g | CV (%) |
|---|---|---|---|---|---|
| 1 | 32.4 | 471,600 a | 9.3 | 112.7 a | 11.4 |
| 2 | 42.0 | 438,200 a | 37.1 | 129.3 a | 17.2 |
| 3 | 55.1 | 332,400 b | 10.3 | 241.3 b | 24.0 |
| 4 | 67.1 | 123,600 c | 46.5 | 86.7 c | 11.3 |
| 5 | 76.7 | 5,400 d | 194.0 | 123.1 a | 5.3 |
| 6 | 81.0 | 2,400 d | 55.9 | 198.9 d | 5.9 |
| 7 | 87.1 | 7,200 d | 63.2 | 94.2 c | 4.6 |

[a]Values are the means of 5 determinations. Means in a column followed by a different letter are significantly different (P < 0.05).

reached 81° C. There were no significant differences in aflatoxin concentrations between grind times of 1 and 5 min and between 4 and 7 min. The CV's for aflatoxin appeared to stabilize at 5 min. These data suggest that differences in aflatoxin concentration were probably associated with subsampling variability and not the temperatures produced during grinding.

*Effect of grinding peanuts with water on temperature of ground samples*

Adding an equal weight of water to peanuts reduced the temperature of the slurry compared with that of peanuts ground alone (Figure 2). Whereas the quantity of *A. flavus/A. parasiticus* recovered was significantly reduced after 3 min of dry grinding at 55.1° C (Table 1), the maximum temperature of the slurry after 7 min was only 46.3° C. The increase in slurry temperature over time was nearly linear ($R^2 = 0.985$; n = 3; P < 0.0001).

*Effect of slurry temperature on survivability of* A. flavus/A. parasiticus

Table 2 illustrates the effect of increasing slurry temperature on survivability of *A. flavus/A. parasiticus*. There were no significant differences in density of *A. flavus/A. parasiticus* recovered between 35° and 45° C. The first significant (P < 0.0001) reduction in *A. flavus/A. parasiticus* was seen when the temperature rose from 45° to 50° C,
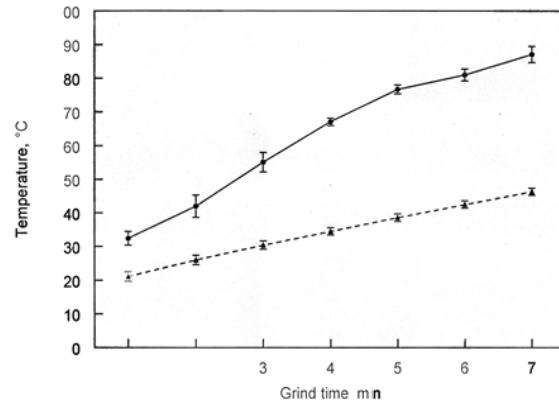


**Figure 2**. Effect of grind time on the temperature of peanuts ground alone ( ● ) and peanuts ground with an equal weight of water (- - - ▲- - -); n = 3, bars indicate standard deviations.

and significant reductions continued to 60° C when less than 1% of the *A. flavus/A. parasiticus* originally found could be recovered.

**Table 2. Quantity of *A. flavus/A. parasiticus* recovered from subsamples of two peanut-water slurries taken at different temperatures during VCM grinding.**

| Temp, (° C) | A. flavus / A. parasiticus Slurry 1 | Slurry 2 |
|---|---|---|
| 35 | 54,667 a | 37,600 a |
| 40 | 49,867 a | 43,600 a |
| 45 | 51,200 a | 35,333 a |
| 50 | 34,533 b | 26,933 b |
| 55 | 20,400 c | 16,493 c |
| 60 | 253 d | 87 d |

[a]Values are the means of 5 determinations. Means in a column followed by a different letter are significantly different (P < 0.05).

*Effect of slurry grind time on* A. flavus/A. parasiticus *and aflatoxin subsample variability*

Means and CV's for *A. flavus/A. parasiticus* colony forming units (CFU)/g and aflatoxin concentrations in peanuts ground with water for various time intervals up to 7 min are presented in Table 3. CV's for *A. flavus/A. parasiticus* were relatively low at all

**Table 3.    Quantity and variability of *A. flavus/A. parasiticus* and aflatoxin associated with subsamples taken from a VCM-ground peanut-water slurry at different time intervals**

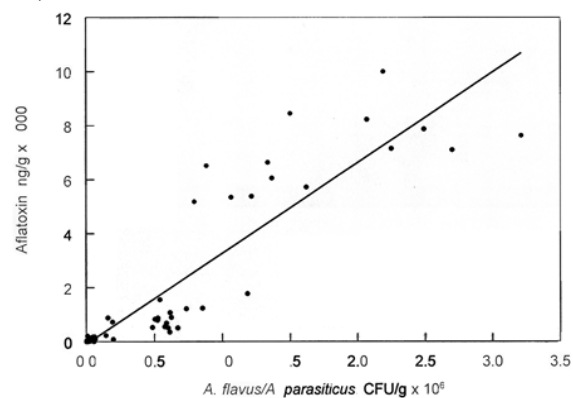| | *A. flavus / A. parasiticus* | | Aflatoxin | |
|---|---|---|---|---|
| | CFU/g [a] | CV (%) | ng/g [a] | CV (%) |
| 1 | 548,400 a | 16.5 | 471.1 a | 83.2 |
| 3 | 572,000 a | 5.1 | 297.6 a | 10.5 |
| 5 | 589,000 a | 9.1 | 358.4 a | 32.9 |
| 7 | 599,000 a | 2.5 | 305.3 a | 3.6 |

[a]Values are the means of 5 determinations. Means in a column followed by the same letter are not significantly different (P > 0.05).

time intervals, and they were particularly low for 3 min and beyond. Aflatoxin variability was high after 1 min but dropped dramatically by 3 min. The relatively high CV of 32.9% at 5 min resulted from one of the five subsamples that contained twice as much aflatoxin as the other four subsamples. When that subsample was not included in the calculations, the CV dropped to 7.6%. The high level of aflatoxin associated with one subsample probably resulted from a relatively larger particle containing a high concentration of aflatoxin in that subsample. The average aflatoxin concentration for the entire sample was about 350 ng/g, whereas *A. flavus/A. parasiticus* averaged about 577,000 CFU/g.

*Correlation between* A. flavus/A. parasiticus *and aflatoxin*

Analysis of 60 samples of peanuts revealed a wide range in quantities of both *A. flavus/A. parasiticus* and aflatoxins. Total aflatoxin concentrations ranged from 0 to 10,000 ng/g and total *A. flavus/A. parasiticus* ranged from $1.4 \times 10^3$ to $3.2 \times 10^6$ CFU/g. Five samples contained no aflatoxin, eighteen contained 1-100 ng/g total aflatoxins, eighteen were in the range of 100-1000 ng/g, and nineteen had > 1000 ng/g. For the 55 samples containing aflatoxins, fourteen contained only aflatoxins $B_1$ and $B_2$. Aflatoxin $B_1$ comprised on average 80.4% of the total aflatoxins for all contaminated samples. Aflatoxins $B_2$, $G_1$, and $G_2$ comprised on average 12.5%, 5.2%, and 1.9%, respectively, of total aflatoxins. *A. flavus* accounted on average for 97.8% of the total *A. flavus* and *A. parasiticus* populations. Whereas *A. flavus* was found in all 60 samples, *A. parasiticus* was not detected in 25 of the samples. Regression analysis showed a highly significant positive correlation (P < 0.0001) between the quantities of total *A. flavus/A. parasiticus* and total aflatoxins present in peanuts (Figure 3; $R^2 = 0.82$; n = 60).



**Figure 3**. Linear regression of aflatoxin concentration vs. quantity of *A. flavus/A. parasiticus* in 60 samples of peanuts (y = 0.00336x - 93.145; $R^2 = 0.82$).

**Discussion**

Peanut samples of varying size can be analyzed with this procedure by using a VCM of appropriate size. Small samples can be processed in a blender, but small samples

introduce greater error. Horn et al. (4) plated whole kernels as well as peanut-water slurries containing 75 g of peanuts to determine the degree of infection of seeds by *A. flavus* and *A. parasiticus*, but aflatoxin concentrations were determined on separate samples. They found that both the whole seed platings and dilution platings of peanut slurries were poor indicators of the amount of aflatoxin. Therefore, it is advisable to use the largest sample size possible and to determine the quantities of both *A. flavus/A. parasiticus* and aflatoxin from the same sample to reduce sampling error. In this study, the Robot Coupe VCM could process samples of up to about 1.5 kg. The Stephan machine, which was larger, could handle samples of up to about 3.8 kg. Other VCM models are available that can process even larger samples.

Water slurries have been used in the past in the preparation of large subsamples of ground peanuts for aflatoxin analysis. In the alternative BF method used for aflatoxin in peanuts (21), 1100 ± 200 g of peanut meal, which has already been comminuted in a subsampling mill, is combined with a volume of water equal to 1.4544 × the peanut-meal weight. The mixture is blended at medium speed for 3 min in a 4 L blender jar, and 196 g of slurry is extracted with 283 mL of methanol. This methodology is used in Agricultural Marketing Service (AMS) labs that monitor shelled peanut lots for aflatoxin prior to sale. However, in that method, large samples (~ 22 kg) of peanuts are first ground in a subsampling mill, and the subsample is further ground with water to produce a slurry before a second subsample is taken for analysis. In the procedure reported here, subsampling error is reduced because only one subsampling step is needed.

Dorner and Cole (20) showed that VCM's produced less subsampling error than the AMS subsampling mill in preparing samples for aflatoxin analysis. However, the temperature of the peanut paste produced in a VCM is sufficiently high to kill most viable propagules of *A. flavus/A. parasiticus* (Table 1). Grinding peanuts with water to produce a slurry reduced the temperature enough so that both aflatoxin and *A. flavus/A. parasiticus* could be quantified from the same samples. In producing the water slurry, *A. flavus/A. parasiticus* propagules were distributed homogeneously faster than was aflatoxin. After 5 min, the CV for *A. flavus/A. parasiticus* was only 9.1% compared with 32.9% for aflatoxin 3). A grind time of 7 min produced very low CV's for both *A. flavus/A. parasiticus* and aflatoxin, and that time is recommended to produce extremely small errors in subsampling. However, it is necessary to ensure that the temperature of the slurry does not exceed about 45° C as there was a significant reduction in viable *A. flavus/A. parasiticus* when the temperature reached 50° C (Table 2).

For aflatoxin extraction, 200 mL of methanol is added to 200 g of the peanut-water slurry. This gives a solvent:peanut ratio of 3:1 with a methanol:water ratio of 67:33. Many commonly used methods for aflatoxin analysis of peanuts use a solvent:peanut ratio of 5:1 with a methanol:water ratio of 60:40 or a solvent:peanut ratio of 2:1 with a methanol:water ratio of 80:20. Cole and Dorner (22) studied the extraction efficiency of different solvents and solvent:peanut ratios and found that the best combination was a solvent:peanut ratio of 3:1 with a methanol:water ratio of 80:20. However, evaluation of those data indicates that the ratios used in this procedure should provide for an efficient extraction of aflatoxin from peanuts.

In application of the method to the analysis of 60 samples of peanuts representing a wide range of contamination, a remarkably high correlation between the quantity of total *A. flavus/A. parasiticus* and aflatoxin was seen. This correlation is noteworthy when

viewed in light of earlier studies that showed no correlation between *A. flavus/A. parasiticus* infection of peanuts and aflatoxin contamination (4, 12-14). Although this procedure gives no indication as to the percentage of peanuts infected with *A. flavus/A. parasiticus*, more importantly, the degree of proliferation of *A. flavus/A. parasiticus* in peanuts is indicated. This could be particularly useful in studies of preharvest aflatoxin contamination when the stress period is insufficient to result in contamination with aflatoxin, but some degree of infection and proliferation by *A. flavus/A. parasiticus* has taken place. For example, in evaluating peanut genotypes for resistance to *A. flavus/A. parasiticus* and aflatoxin contamination, the degree of *A. flavus/A. parasiticus* proliferation could provide very useful information when aflatoxin has not formed or aflatoxin results are inconsistent.

Use of this method also allows for the determination of the degree of proliferation in peanuts by the individual species, *A. flavus* and *A. parasiticus*, as well differentiation among strains of the individual species. Although data for the two species were totaled for simplicity of reporting in this study (*A. parasiticus* accounted for only 2.2% of the total), the species were differentiated easily on the medium used for dilution plating (5). The method also has been used to determine the ratio of toxigenic to nontoxigenic strains of *A. flavus* in peanuts from aflatoxin biocontrol studies. This provides a direct measure of the competitive exclusion that is achieved by applying competitive, nontoxigenic strains of *A. flavus* and *A. parasiticus* to soil (23).

## Acknowledgments

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